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Quantitative and Molecular Analyses of Genetic Risk: A Study with Ionizing Radiation

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Mammalian cells in culture have been used to study the genetic effects of physical and chemical agents. We have used Chinese hamster ovary (CHO) cells, clone K1-BH4, to quantify mutations at the X-linked, large (35 kb) *hypoxanthine-guanine phosphoribosyltransferase (hprt) locus (the CHO/HPRT assay)* induced by environmental agents. By transfecting an *hprt*-deletion mutant CHO cell line with the plasmid vector pSV$_2$*gpt*, we isolated a transformant, AS52. AS52 cells carry a single functional copy of an autosomal, small (456 bp) xanthine-guanine phosphoribosyltransferase (*gpt*) gene (the bacterial equivalent of the mammalian *hprt* gene; AS52/GPT assay). We found that ionizing radiations such as X-rays and neutrons and oxidative genotoxic chemicals such as Adriamycin, bleomycin, hydrogen peroxide, and potassium superoxide are much more mutagenic to the *gpt* gene in AS52 cells than to the *hprt* locus in K1-BH4 cells. The hypermutability of the *gpt* gene probably results from a higher recovery of multilocus deletion mutants in AS52 cells than in K1-BH4 cells, rather than a higher yield of induced mutants. These results demonstrate that the use of the *hprt* locus alone could lead to an underestimate of the genetic risk of these agents. Analyses of the mutation spectrum using a polymerase chain reaction-based deletion screening and DNA sequencing procedure showed that a high proportion of HPRT$^-$ and GPT$^-$ mutants induced by X-rays carry deletion mutations. Thus, both the mutant frequency and mutation spectrum need to be considered in assessing the genetic risk of ionizing radiation and oxidative genotoxic chemicals.

Introduction

Sixty-five years ago, Muller discovered that X-rays induce mutations in *Drosophila melanogaster* (*1*). Since then, the genetic effects of radiation have been extensively studied in a wide spectrum of biological systems (*2,3*). Over the past quarter of a century, several mammalian cell mutational systems have been developed for studying chemical and radiation mutagenesis (*4,5*). These include the endogenous hypoxanthine-guanine phosphoribosyltransferase (*hprt*), adenine phosphoribosyltransferase (*aprt*), thymidine kinase (*tk*), and the transgenic xanthine-guanine phosphoribosyltransferase (*gpt*) gene.

Most studies on the mutagenic effects of ionizing radiation are concerned with the induction of mutant frequency at these genetic loci. Quantitative analyses of radiation-induced mutant frequency have been used to assess the potential genetic risk imposed by radiation. Recent advances of mammalian molecular genetics has enabled analyses of radiation-induced gene mutations at the DNA sequence level.

In this paper, we present aspects of our quantitative and molecular studies of radiation-induced mutations in the *hprt* locus in Chinese hamster ovary (CHO) cells clone K1-BH4 (CHO/HPRT assay [*6*]) and in the *gpt* gene in a CHO cell derivative, AS52 (AS52/GPT assay [*7*]). Evidence is presented to demonstrate that both the mutant frequency and the molecular spectrum of gene mutations need to be considered in the assessment of radiation-induced genetic risk.

Materials and Methods

Cell Lines, Culture Conditions, and Radiation Mutagenesis

K1-BH4 and AS52, a subclone and derivative of the CHO-K1 cell line, respectively, were used in all the experiments to be described. Whereas K1-BH4 cells contain an endogenous *hprt* gene (*6*), the AS52 cells contain a transgenic *gpt* gene (*7*). Cell cultures were maintained in Ham's

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F12 medium containing 5% fetal calf serum (F12FCM5) in a 5% CO_2-95% air incubator at 37°C with 100% humidity. To decrease the background of $HPRT^-$ mutants, K1-BH4 cells were treated with F12 medium containing aminopterin (HAT medium), and to decrease of the background of GPT^- mutants, AS52 cells were treated with F12 medium containing adenine, aminopterin, and mycophenolic acid (MPA medium) for 2 days as we described earlier (*6,7*). Both cell types were then grown in F12FCM5 medium for another 2 days before irradiating these cells with X-rays (*8,9*).

Our previously published procedure was used to determine both the radiation-induced cytotoxicity and mutagenicity (*10*). Cytotoxicity was expressed as percent of surviving clonable cells relative to that of untreated control(s). Mutant frequency was calculated as the number of 6-thioguanine-resistant (TG^r) colonies per 10^6 clonable cells at the end of 7 of days phenotypic expression time. Independent TGr mutants were isolated (*9,11*) for the analysis of the molecular spectrum of gene mutations described below.

Molecular Analyses of the Mutation Spectrum at the *hprt* and *gpt* Loci

For the analysis of the molecular spectrum of radiation-induced mutations at the *hprt* locus, our recently developed polymerase chain reaction (PCR)-based comprehensive procedure was used (*9,11*). This procedure includes direct sequencing of PCR-amplified *hprt* cDNA for locating point mutations in the expressed coding sequences, multiplex PCR-amplification of all nine *hprt* exons for screening large deletions, and direct sequencing of PCR-amplified *hprt* exons and their flanking regions for detecting intronic mutations resulting in mRNA splicing errors. To analyze the mutation spectrum of the *gpt* gene in AS52 cells, we have adapted the nested PCR amplification method (*9,12*).

Results and Discussion

CHO/HPRT Mutation Assay

In 1974, we used CHO cells, clone K1-BH4, to develop a quantitative mutation assay at the *hprt* locus, the CHO/HPRT assay (*6*). The quantitative nature of this assay has been used to determine the mutagenicity of radiation and chemicals and to study mechanisms of mammalian cell mutagenesis (*4,13,14*).

In our studies using the CHO/HPRT assay with physical agents, we found that ultraviolet (UV) light is a strong mutagen; it causes a linear dose-dependent increase in mutagenicity (*15*). However, X-rays are relatively weakly mutagenic. The mutant frequency was found to increase from approximately 30×10^{-6} mutants per cell at 100 rads to 85×10^{-6} mutants per cell at 600 rads (with a spontaneous mutant frequency of 9×10^{-6} mutants per cell). The shape of the dose–response curve could not be defined adequately (*16*). One possible explanation for the apparent weak mutagenic response of CHO K1-BH4 cells to X-rays could be a low recovery of multilocus deletion mutants induced by X-rays rather than a weak mutation induction per se (*17*).

AS52/GPT Assay

In 1982, we transformed an X-ray-induced, *hprt*-deletion subclone K1-BH4 cells with a plasmid vector, pSV_2gpt. The *gpt* gene, which codes for the enzyme GPT, is the bacterial equivalent of the mammalian *hprt* gene. We obtained a transformant, AS52, that carries a single functional copy of the *gpt* gene stably integrated into the high-molecular-weight DNA of the host (*7*). The AS52 cell line fulfills the classical requirements for specific-locus mutation assays in mammalian cells (*18*).

We found that X-irradiation is equally toxic to both AS52 cells and the parental K1-BH4 cells. However, X-rays are approximately 10 times more mutagenic to the *gpt* gene in AS52 cells than to the *hprt* locus K1-BH4 cells (Fig. 1). Thus, AS52 cells are hypersensitive to mutations induced by X-rays. Southern blot analysis showed that the proportion of the TG-resistant mutants induced by X-rays that carry a total loss of the *gpt* gene in AS52 cells is much higher than that of the *hprt* gene in K1-BH4 cells. Small deletions are the most frequent type of deletion in spontaneous mutants (*8,9,12*).

Because X-irradiation induced predominantly deletion mutations in these cells as analyzed by Southern blot hybridization (*8*), the observed low mutagenic activity of X-irradiation in K1-BH4 cells, where the *hprt* gene exists in a hemizygous state, is likely due to lethal events associated with multilocus deletions, reducing the viability of the induced TG-resistant mutants. The *gpt* gene is most likely integrated in the heterozygous state in one of the autosomes of AS52 cells. In AS52 cells, a multilocus deletion induced by X-irradiation could produce only a hemizygous state in a vital gene that flanks the *gpt* gene, resulting in a higher probability of recovering induced TG-resistant mutants rather than to an actual reduced mutation induction (*17,19,20*).

We found that neutrons are also mutagenic to the *hprt* gene in CHO cells. The mutant frequency was found to increase from approximately 35×10^{-6} mutants per cell at 20 rads to 100×10^{-6} mutants per cell at 100 rads (with a spontaneous mutant frequency of 12×10^{-6} mutants per cell). The pattern of X-ray-induced differential mutagenic response between the *hprt* locus in K1-BH4 cells and *gpt* gene in AS52 cells was also produced by irradiation with neutrons. Neutron irradiation resulted in nearly identical toxicity to both cell types; however, neutrons were approximately 10 times more mutagenic to the *gpt* gene than to the *hprt* locus (*17*).

Evidence for Reactive Oxygen Species Inducing Mutations

Reactive oxygen species (ROS) are being implicated in the toxic action of ionizing radiation and oxidative chemicals (*21*). If ROS were to mediate the mutagenic effects of

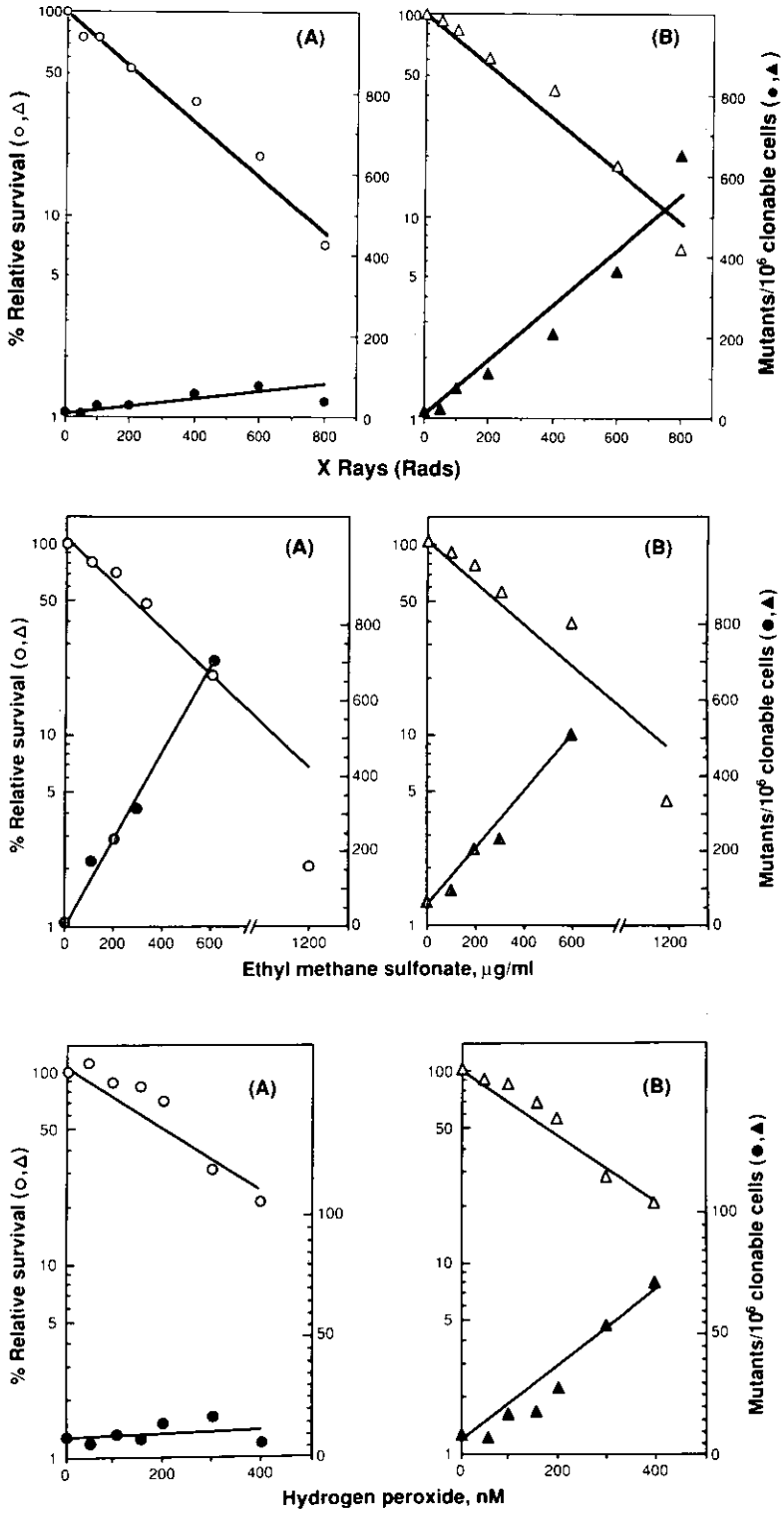

FIGURE 1. The cytotoxic (○, △) and mutagenic (●, ▲) effects of X-rays, ethyl methanesulfonate, and hydrogen peroxide. (*A*) Percent relative survival; (*B*) mutants/10^{-6} clonable cells.

radiation such as X-rays and neutrons, then oxidative genotoxic chemicals such as streptonigrin, Adriamycin, and bleomycin, which are known to produce superoxide and hydroxy radicals, would be expected to be equitoxic to both cell types and more mutagenic to the *gpt* gene in AS52 cells than to the *hprt* locus in K1-BH4 cells. Likewise, potassium superoxide and hydrogen peroxide, which are ROS themselves, should be more mutagenic to the *gpt* gene in AS52 cells and equitoxic to both cell types. The results of our experiments fulfilled such expectations (Fig. 1). We have previously shown that agents such as ethyl methanesulfonate (EMS), ICR-191, and W-light, which do not produce ROS, do not elicit differential mutagenic response in both cell types (*8,22*) (Fig. 1). Taken together, these experiments support the view that one of the mechanisms responsible for the mutagenic effects of ionizing radiation and oxidative genotoxic chemicals is mediated through ROS (*17,19,20*).

Molecular Analyses of Gene Mutations

Southern blot analysis shows that ionizing radiation induces predominantly deletions at the *hprt* locus in CHO cells clone K1-BH4 (*8*). Mutations undetectable by Southern blot analysis have largely been uncharacterized because appropriate large-scale molecular genetic pro-

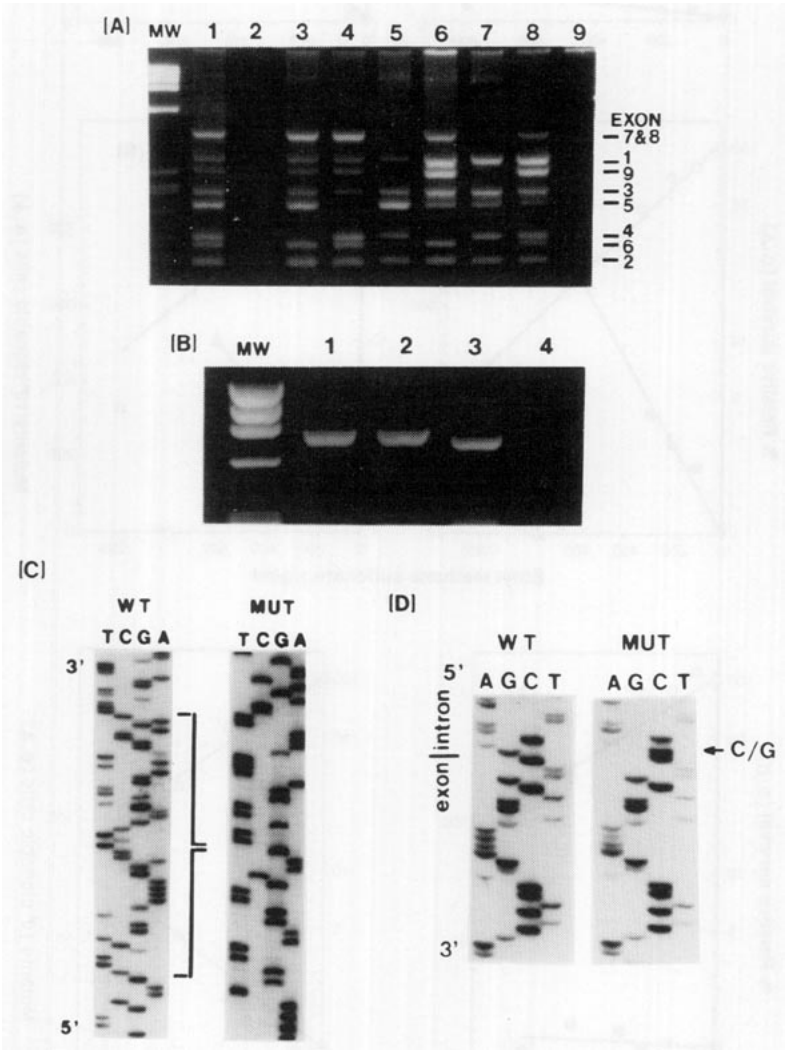

FIGURE 2. Molecular analysis of mutation spectra of the *hprt* locus in CHO cells. (*A*) Multiplex exon amplification for deletion screening. All nine *hprt* exons were simultaneously amplified in a single PCR. Exons 7 and 8 were amplified in one DNA fragment. (Lane 1) standard *hprt* positive control (K1-BH4 cells); (lane 2) negative control (no DNA template); (lanes 3–5) spontaneous mutants (lane 3, exon 4 deletion; lane 4, exon 5 deletion; lane 5, exons 6–9 deletion); (lanes 6–9) γ-ray-induced mutants (lane 6, exon 4 deletion; lane 4, exons 6–9 deletion; lane 8, intact exons; lane 9, a total deletion). (*B*) PCR amplification of the *hprt* cDNA. A 841-bp DNA fragment containing the entire peptide coding region (651 bp) of the *hprt* mRNA was amplified via PCR from cDNA of the parental K1-BH4 cells (lane 1), a mutant with base-substitution (lane 2), and a mutant with a deletion of 47 bp (lane 3). No cDNA fragment was observed on one mutant (lane 4). (*C*) Direct sequence analysis of PCR-amplified *hprt* cDNA. A deletion of entire exon 7 coding sequence (48 bp; exon skipping) was identified in the *hprt* cDNA of a mutant. (*D*) Direct sequencing of PCR-amplified individual exons of the *hprt* gene. A transversion of G to C in the splice acceptor site of exon 7 in a mutant which causes exon 7 skipping in cDNA.

cedures to analyze the mutation spectrum of the *hprt* locus at the DNA sequence level have been developed only recently (*9,11*). A preliminary analysis of spontaneous mutations of the *gpt* gene in CHO cell subclone AS52 showed that a high proportion of the spontaneously arising mutations are deletions (*12*).

Recently, we established a PCR-based comprehensive procedure for molecular analysis of mutation spectrum at the *hprt* locus in CHO cells, clone K1-BH4 (*9,11*).This procedure includes *a*) multiplex PCR amplification of all nine *hprt* exons for deletion screening, *b*) direct sequencing of PCR-amplified *hprt* cDNA for identification of point mutations at the coding region, and *c*) direct sequencing of PCR-amplified individual exon and flanking regions for determining splicing mutations (Fig. 2).

Using this PCR procedure, we have analyzed the mutation spectrum of 63 independent spontaneous HPRT$^-$ mutants in K1-BH4 cells. We found that there exists a wide spectrum of mutational events among these mutants (Table 1). These include single base substitution (41%), deletions (25%), RNA splicing errors (25%), frameshift ($\pm$ 1 bp; 3%), insertion (2%) and gene rearrangements (3%). Two hot spots appear to exist for single base substitution. The majority of deletion breakpoints (71%) were found in regions around exons 4, 5, and 6. RNA splicing errors were found to affect exons 3 and 9, and mostly resulted in the loss of exon 7 (40%) (*9,11*).

Results from the sequence analysis of 43 spontaneous GPT mutants in AS52 cells showed that the great majority (68%) of mutations is deletion. Among these deletion mutants, 76% have lost the entire *gpt* gene. Other mutation types include 14% single base substitution, 9% frameshift ($\pm$ 1 bp) and 9% insertion (9). We found a 3-bp deletion hot spot as reported by others (*12*).

We screened for X-ray-induced deletions and found that X-rays induce primarily deletions (*9*). Among 41 HPRT$^-$ mutants induced by X-rays at 400 rads, 51% of the mutants had lost the entire *gpt* gene. Analyzing 28 mutants induced by 600 rads of X-rays, the proportion of total deletion increased to 68%. It appears that the induction of deletion-type mutations could be dose dependent. Seven percent of the HPRT$^-$ mutants induced by X-rays at 600 rads exhibits partial deletion (Table 2). Analyses of 25 GPT$^-$ mutants induced by X-rays (400 rads) showed that 80% of them have lost the entire *gpt* gene and another 4% exhibits a partial deletion (Table 2).

These data on X-ray-induced mutation spectrum of both the *hprt* and *gpt* gene (*9*) are consistent with our earlier findings from cellular mutagenesis in which we found that the majority of X-ray-induced mutants at these two loci were found to be deletion as analyzed at the cellular level (*17,19,20*).

Quantitative and Molecular Analyses of Genetic Risk

Our studies demonstrate that ionizing radiations and oxidative genotoxic chemicals, both of which are known to generate ROS as a major mechanism, are approximately

Table 1. Spectrum of spontaneous mutations.

Type of mutation	CHO K1-BH4/*hprt*	CHO AS52/*gpt*
Deletion	25% (16/63)	68% (29/43)
Total deletion	(0/16)	(22/29)
Exon deletion	(14/16)	NA
Base pair deletion (>2 bp)	(2/16)	(7/29)
Single base substitution	41% (26/63)	14% (6/43)
Transition	(7/26)	(4/6)
Transversion	(19/26)	(2/6)
Frameshift ($\pm$ 1 bp)	3% (2/63)	9% (4/43)
Insertion	2% (1/63)	9% (4/43)
RNA splicing mutation	25% (16/63)	NA
Others	3% (2/63)	NA

Table 2. Deletion screening of X-ray-induced mutations.

Type of mutation	CHO K1-BH4/*hprt*		CHO AS52/*gpt*,
	400 rad	600 rad	400 rad
Total deletion	51% (21/41)	68% (19/28)	80% (20/25)
Partial deletion		7% (2/28)	4% (1/25)
Rearrangement		4% (1/28)	
Nondetectable change	49% (20/41)	21% (6/28)	16% (4/25)

5–10 times more mutagenic to the heterozygous *gpt* gene than to the hemizygous *hprt* locus. Thus, these agents could be considered a potent mutagen to one genetic marker (*gpt*) and a weak mutagen to another (*hprt*).

Demonstration that a high proportion of mutants induced by radiation and ROS producers may not survive to be scored as mutants in the K1-BH4 cells (CHO/HPRT assay) implies that assessment of genetic risk for these agents using hemizygous genetic markers such as *hprt* might be underestimated. This also leads to consider a need to reevaluate assessment of genetic risk relative to ionizing radiation. Our studies on the relationship between mutation induction and mutant recovery as analyzed by Southern blot hybridization and PCR procedure suggests a role of molecular genetics in the quantitative assessment of the mutagenic risk.

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